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13. ABSTRACT (Maximum 200 Words) Population studies have shown that women who use non-steroidal anti-inflammatory drugs (NSAIDs) develop breast cancer less frequently. However, these drugs have side effects toward the stomach, liver and kidneys, particularly at the high doses potentially required to prevent breast cancer. Also, how these agents prevent breast cancer is not understood. This project will develop an optimized NSAID for breast cancer prevention that can be taken safely at high doses, and will determine its mechanisms of action. The side effects of NSAIDs are mainly due to inhibition of the cyclo-oxygenase (COX) enzyme. Based on preliminary experiments, we hypothesize that the preventative action of NSAIDs in breast cancer is not due to COX inhibition, but rather to alterations of other biochemical pathways in breast cells that control their proliferation. We have isolated modified NSAIDs that do not inhibit COX inhibitors, but still retain chemopreventative activity. We will study genetically modified mice that frequently develop breast cancer. The mice will be fed diets supplemented with placebo or with the modified NSAID that does not inhibit COX. We shall determine if the new agent prevents breast cancer without toxicity to normal cells, and will measure biochemical parameters associated with its proposed mechanisms of action.				
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Introduction

There is an unmet need for agents that can prevent the development of estrogen receptor negative breast cancer. A characteristic feature of these tumors is the high levels of expression of cyclin D1, that is an established target of the wnt oncogenic pathway. Indeed, MMTV-wnt1 transgenic mice develop breast cancer, in the absence of a functional estrogen receptor. In preliminary experiments, we have found that particular compounds (e.g., R-eto) related to the non-steroidal anti-inflammatory drugs, but devoid of the gastrointestinal side effects, due to cyclo-oxygenase inhibition, nonetheless can block wnt signaling in cancer cells at concentrations that are achievable *in vivo* after oral administration. Therefore, we hypothesize that these agents will be able to prevent breast cancer in the MMTV-wnt1 transgenic mouse model. The proposed experiments will test this supposition. The work is innovative, because no safe inhibitors of wnt signaling are currently known. If successful, the results will be clearly relevant to the needs of women at risk for breast cancer.

Body

Task 1. To determine if supplementation with R-eto can prevent the development of breast cancer in the MMTV-wnt1 transgenic model.

a. Increase size of breeding colony of FVB/NJ-TgN[Wnt]1 Heu mice, and confirm expression of the transgene in at least 50 female offspring (Months 1-6).

To date 54 MMTV-wnt1 positive transgenic females have been bred and randomized to receive R-eto or control chow.

b. Divide mice into drug-treated and control groups, and observe for breast cancer development (Months 7-17).

Observation for breast tumor development is ongoing. To date 15 mice (9 control and 6 R-Eto feed) have developed tumors (see Figure 1, Kaplan-Meier graph.)

Figure 1

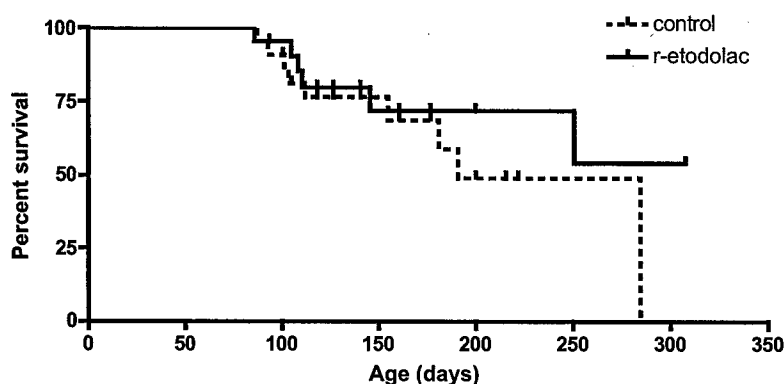


Figure 1 – Kaplan-Meier Survival Curves for MMTV-wnt1 transgenic mice on control versus R-Etodolac Chow.

c. Perform pathologic and molecular analyses of breast tumors from mice in both groups (Months 12-20).

Task not due at this time. A preliminary result utilizing terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) reveals increased apoptosis in treated tumors (Figure 2.)

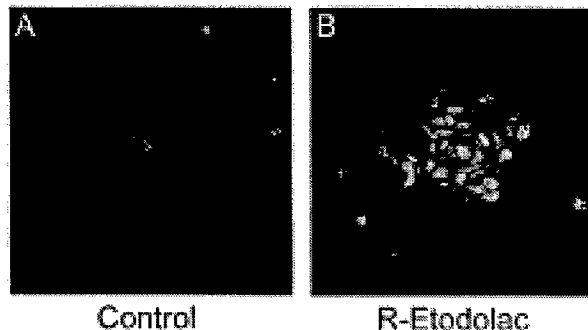


Figure 2 - TUNEL of breast cancers from control versus R-etodolac treated MMTV-wnt1 transgenic mice. Images were taken using a DeltaVision Deconvolution Microscope at a magnification of 400X.

d. Repeat treatment protocol in independent groups of mice, with dosage modification if indicated by first experimental data set (Months 21-30).

Task not due at this time.

e. Repeat analyses of excised breast tumors (Months 31-33).

Task not due at this time.

f. Complete supportive biochemical and pharmacologic studies (Months 34-36).

Task not due at this time.

Task 2. To determine the mechanism of action of the modified non-steroidal anti-inflammatory drug R-eto in mammary tissues, emphasizing the regulation of wnt signaling.

a. Optimize protocol for quantitative gene expression of wnt-related genes in primary mammary tissues (Months 1-6).

A commercially available kit, the Qiagen RNeasy Kit, was used to extract RNA from flash frozen Wnt1 breast tumors from control and treated mice. Tumors were pulverized with mortar and pestle while maintained at less than -80 degrees Celsius prior to extraction. Purified RNA was utilized in Affymetrix gene chip arrays through the UCSD Cancer Center Genechip Core. Table 1 details preliminary results of this experiment.

Table 1 – Change in gene expression with R-Eto treatment.

Fold reduction

- 11 - Casein alpha
- 10 - Protein Phosphatase 1, catalytic subunit, beta isoform
- 8 - Peroxiredoxin 1
- 7 - Bcas1
- 7 - Acid phosphatase 1, soluble

Fold Induction

- 17.5 - Major urinary protein 1
- 11 - Collagenous repeat-containing sequence
- 11 - Retinitis pigmentosa GTPase regulator interacting protein 1
- 8 - Adipsin
- 8 - Adipocyte complement related protein of 30 kDa (Acrp30)
- 6 - Carbonic anhydrase 3
- 6 - Fatty acid binding protein 4

Table 1 – Comparison of gene expression between MMTV-wnt1 breast tumors developing in mice on control versus R-Eto chow.

b. Optimize immunohistochemical procedures for assessing wnt-signaling in primary tumors (Months 1-6).

Antibodies directed against Wnt1 are not effective in immunohistochemistry. This is generally believed to be related to Wnt1's tight association with extracellular matrix. Therefore to assess wnt-signaling, immunohistochemical assays for a known downstream target (Cyclin D1), suspected drug targets (PPAR γ and RXR α), and a standard marker of proliferation (Ki67) were optimized in breast tumors from treated and untreated MMTV-wnt1 transgenic mice. Tumors were formalin-fixed and paraffin-embedded prior to sectioning to avoid sectioning artifact, a common problem in tissues with high fat content. Sections were then deparaffinized by a standard procedure and antigen retrieved with DAKO antigen retrieval solution[1]. Polyclonal antibodies that react with murine antigens were used for each of the following assays; anti-PPAR γ antibody (ABCAM 12410), anti-RXR α antibody (Santa Cruz Biotechnology D20), anti-Ki67 antibody (Novocastra NCL-Ki67p) and anti-cyclin D1 antibody (biosource AHF0102.) Preliminary results revealing chemomodulation PPAR γ and RXR α along with downregulation of Cyclin D1 and Ki67 are shown in **Figure 3**.

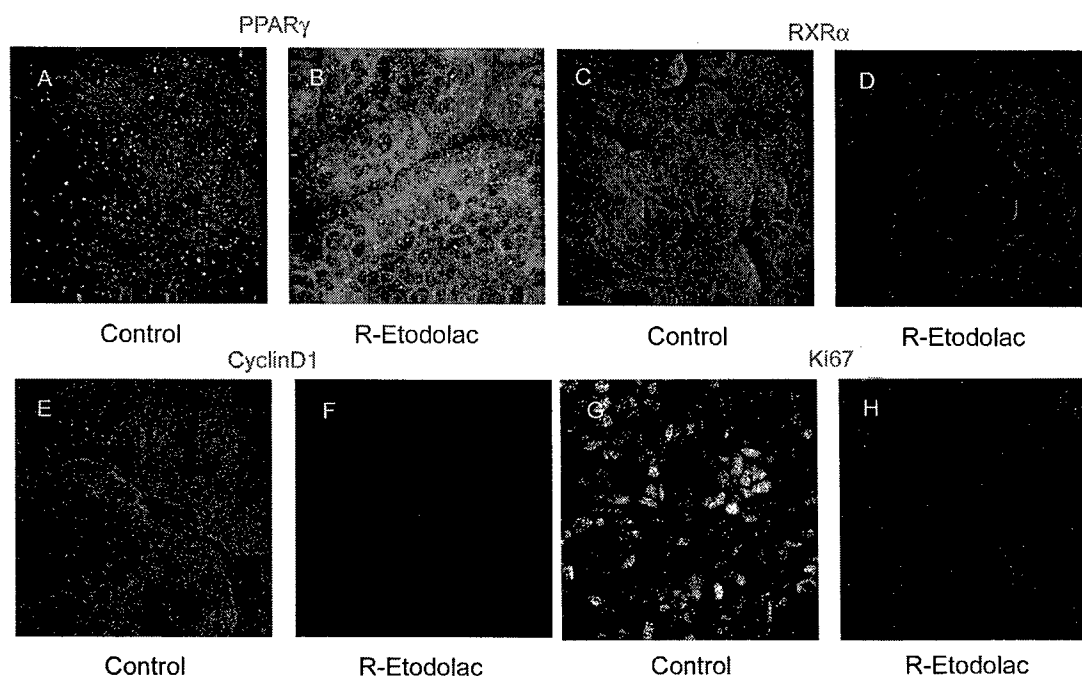


Figure 3- Immunohistochemical stain of breast tumors from MMTV-wnt1 transgenic mice. Transgenic MMTV-wnt1 mice that developed nodules while fed control versus R-etodolac supplemented chow. The mice were sacrificed and the tumors were excised, fixed and embedded. Histologic sections were stained with the indicated antibodies. Images were taken using a DeltaVision Deconvolution Microscope at a magnification of 400X.

c. Complete analyses of gene and protein expression in tumors from MMTV-wnt1 transgenic mice (Months 7-36).

Task not due at this time. Please see task 2a and 2b for preliminary results.

d. Complete wnt signal transduction studies in breast cancer cells treated with R-eto and other NSAIDs (Months 1-36.)

Task not due at this time. Wnt signal transduction studies are first being optimized and completed in standard cell culture systems for R-eto and other NSAIDs prior to transitioning into a breast cancer cell system.

Key Research Accomplishments

- A trend towards breast tumor prevention is seen with R-eto even at an early time point with a small cohort of MMTV-wnt1 transgenic mice, a robust model of breast cancer.
- Oral treatment with R-eto alters gene and protein expression in breast tumors from MMTV-wnt1 transgenic mice.
- R-eto appears to increase apoptosis and decrease proliferation in breast tumors from MMTV-wnt1 transgenic mice.

Reportable Outcomes

Abstract

An abstract describing early immunohistochemical findings from these studies has been submitted and accepted[2].

Presentations

On March 7th, 2005 preliminary data from these studies was presented at the Moores UCSD Cancer Center Translational Conference. This venue is designed to bring basic scientists and clinical researchers together to facilitate the rapid incorporation of scientific advances into clinical practice.

Funding applications

Based on preliminary data from these studies grant applications have been submitted to the American Cancer Society, the California Breast Cancer Research Program, the NCI/Avon "Progress for Patients" Awards, and the Amgen Oncology Institute Hematology and Oncology Fellowship Program.

Conclusions

These studies are based on the hypothesis that a COX-inactive NSAID (R-eto) can specifically block the wnt oncogenic pathway in breast cancer, without host toxicity. Preliminary data reveals downregulation of wnt signaling and a trend towards reduction in breast tumor formation. These studies have taken on even greater importance as similar efforts to develop minimally toxic medications to reduce the risk of ER negative breast cancer have failed. In particular, early studies of COX2 inhibitors as chemopreventative agents while promising[3] have been halted due to newly identified cardiac toxicity[4-6]. These studies could provide evidence to bring a COX-inactive NSAID with chemopreventative activity into clinical trials for women at risk of developing ER negative breast cancer.

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Appendices

Abstract submitted to Era of Hope 2005 meeting

R-ETODOLAC DECREASES β -CATENIN, CYCLIN D1 AND KI-67 EXPRESSION, *IN VIVO*, IN MAMMARY TUMORS FROM MMTV-WNT1 TRANSGENIC MICE

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Epidemiologic studies have shown that the risk of breast cancer for non-steroidal anti-inflammatory drug (NSAID) users is 13 to 22% less than that for non-users. The mechanism, or mechanisms, of action remain controversial. Etodolac is a unique commercially available NSAID that is a racemic mixture in which the R-enantiomer has no COX inhibitory activity and therefore none of the toxicities typically associated with NSAIDs. However, *in vitro* and *in vivo* testing of R-etodolac demonstrates anti-tumor/pro-apoptotic activity in prostate carcinoma and chronic lymphocytic lymphoma. Additional *in vitro* study strongly implicates down regulation of the Wnt/ β -catenin pathway as its mechanism of action. Based on prior evidence of dysregulated Wnt/ β -catenin signaling in breast carcinoma, and additional preliminary data, we are examining R-etodolac's effect on breast carcinoma in the MMTV-Wnt1 transgenic mouse model of breast carcinoma.

MMTV-Wnt1 transgenic mice (strain name FVB/NJ-Tg(Wnt1)¹Hev/J) were purchased from Jackson Laboratory (Bar Harbor, ME) and a breeding colony established. Mice confirmed heterozygous for the transgene were randomly assigned to receive chow with R-etodolac (1.25mg/kg) or control chow starting at a mean age of 7.45 weeks (range 5-10 weeks.) Initially, mice receiving control chow had their primary tumors resected and were then treated with R-etodolac chow. Immunofluorescent staining for β -catenin, cyclin D1 and KI-67 was performed on tumor tissue and analyzed by deconvolution microscopy.

Initial experiments reveal expression patterns typical of malignant tissue for β -catenin, cyclin D1 and KI-67 in tumors from mice on control chow. However dramatically lower levels of β -catenin, cyclin D1 and KI-67 expression was seen in tumors that grew in mice receiving R-etodolac chow. Close monitoring of treated animals and necropsy of sacrificed animals revealed no toxicity associated with R-etodolac treatment.

In vivo testing of R-etodolac in the MMTV-Wnt1 transgenic mouse model of breast carcinoma reveals dramatic inhibition of the Wnt/ β -catenin signaling pathway. Ongoing study of R-etodolac in this model system will lead to (1) further molecular characterization of R-etodolac's mechanism of action (2) characterization of R-etodolac resistance mechanisms in breast carcinoma (3) the generation of preclinical data regarding R-etodolac's efficacy in chemoprophylaxis of breast carcinoma and (4) potentially a biomarker to use in clinical testing. Development of minimally toxic drugs, like R-etodolac, may lead to better therapy for breast cancer patients or successful prophylactic treatment of women at high risk for breast cancer.

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